Separation of the 4 Enantiomers of the Fungicide Spiroxamine by LC-MS/MS

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The development of a chiral LC-MS/MS method to separate the 4 enantiomers of the fungicide Spiroxamine is described. First, a chiral phase screening was performed to identify the most suitable column. The immobilised column CHIRAL ART Amylose-SA showed the best results in the screening and further method development for UV detection was performed using this column. In order to transfer the method to LC-MS/MS some method adaption had to be done. The final LC-MS method takes 20 minutes and allows quantitation of each enantiomer in trace concentrations, as low as 0.23 µg/L.

Introduction

Many pesticides, which have been commonly used for several years, are chiral compounds. Companies which need to re-register these for existing markets or to register them for new markets have to provide exact data on the amounts of enantiomers in the final product. Therefore, chiral methods have to be available to estimate the enantiomeric excess (EE). Often there are no methods or only old methods available, which were developed several years ago using the chiral column available at that time [1]. Therefore, new methods have to be developed using more modern chiral stationary phases with shorter run times and other detection options such as mass spectrometry.

In this article the development of a new state-of-the-art LC-based chiral separation method that can be coupled to MS/MS for the fungicide Spiroxamine is presented. This also required the background of analyses of residues of Spiroxamine at trace concentrations. Therefore, this suggested an investigation with preference for RP conditions. In addition, the goal was to perform the separation of the four isomers in less than 30 minutes to allow a high throughput. It was intended to first develop a separation method using LC-UV and then to transfer the application to LC-MS/MS.

Spiroxamine is a systemic fungicide, which was brought to the market by Bayer AG. The substance is a mixture of diastereomers A and B each of which consists of 2 enantiomers giving 4 enantiomers in total; A1, A2, B1 and B2 (Figure 1).

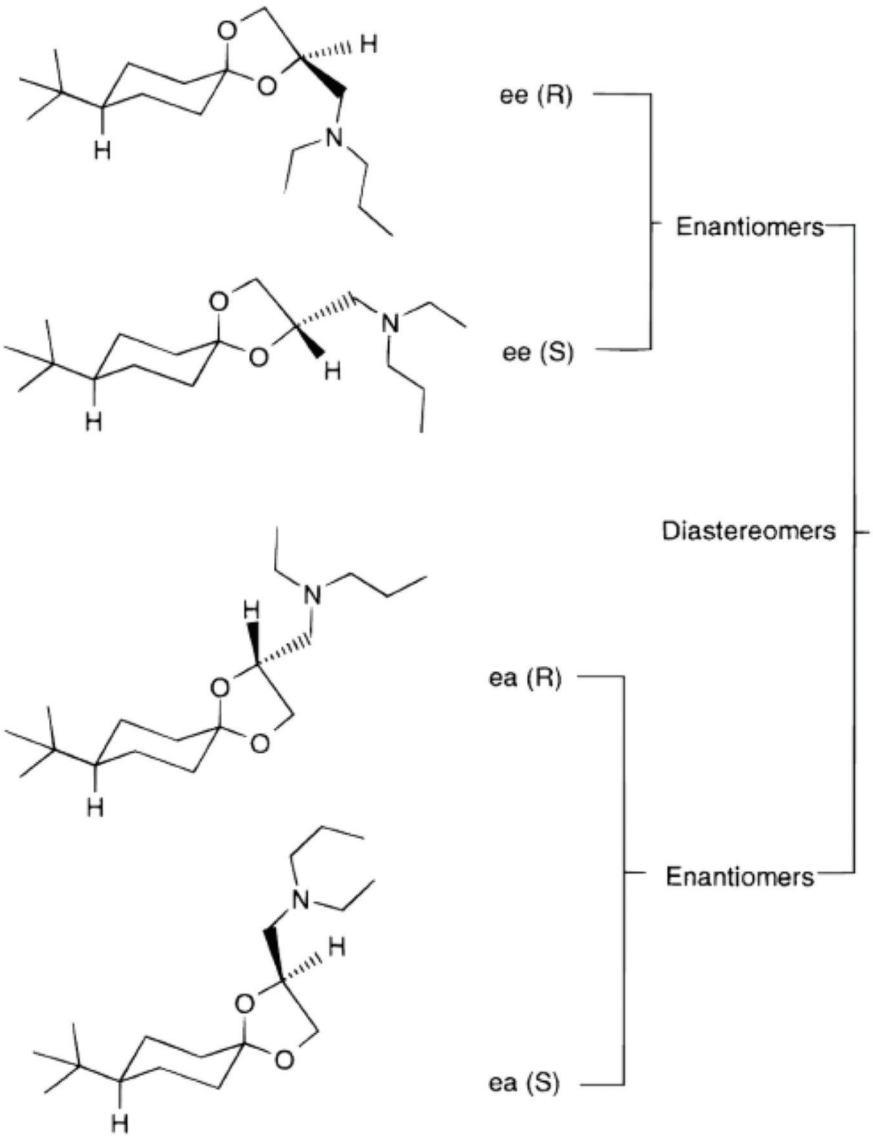

Figure 1: Isomers of Spiroxamine [1], diastereomer A: K_{ow} = 2.79 (at 20°C), diastereomer B: K_{ow} = 2.92 (at 20°C), pKa value = 6.9.

Table 1: Possible mobile phases for the RP mode

Mobile Phase Organic	pH (aq.), typical additive
Acetonitrile	Acidic (pH 2.9), 0.1% HCOOH
Methanol	Neutral (pH 6.9), 10 mM CH_3COONH_4
2-Propanol	Alkaline (pH 9.0), 20 mM NH_4HCO_3
THF	adjust pH 9 with DEA

Table 2: Chiral stationary phases used

Specifications	**CHIRAL ART Amylose-SA**	**CHIRAL ART Cellulose-SB**	**CHIRAL ART Cellulose-SC**
Base particle	silica		
Chiral selector	Amylose tris (3,5-dimethylphenyl-carbamate)	Cellulose tris (3,5-dimethylphenyl -carbamate)	Cellulose tris (3,5-dichlorophenyl -carbamate)
Particle sizes	3, 5, 10, 20 µm		
Type	Immobilised type		
Separation modes	NP/ RP/ SFC		

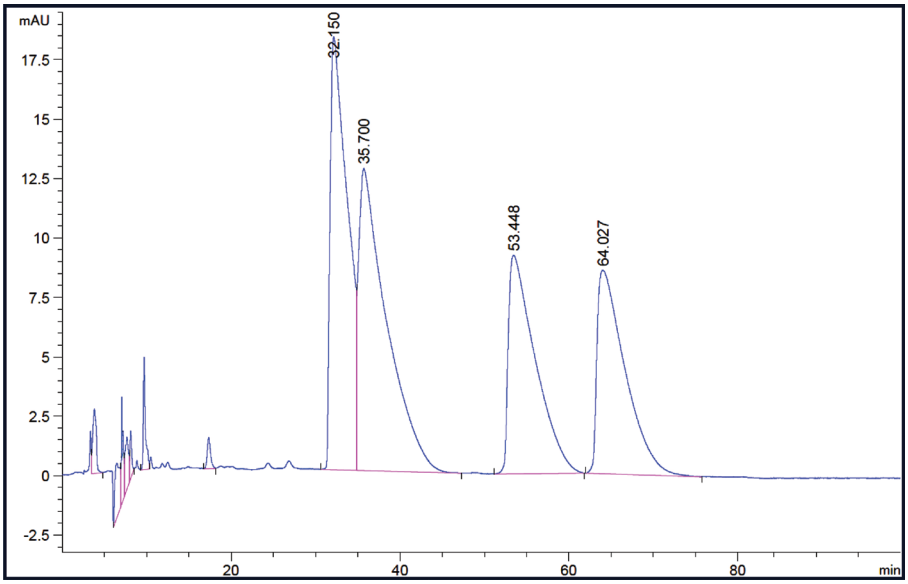

Figure 2: Result for the chiral phase screening of Spiroxamine that was used for optimisation using a CHIRAL ART Amylose-SA, 5 µm column.

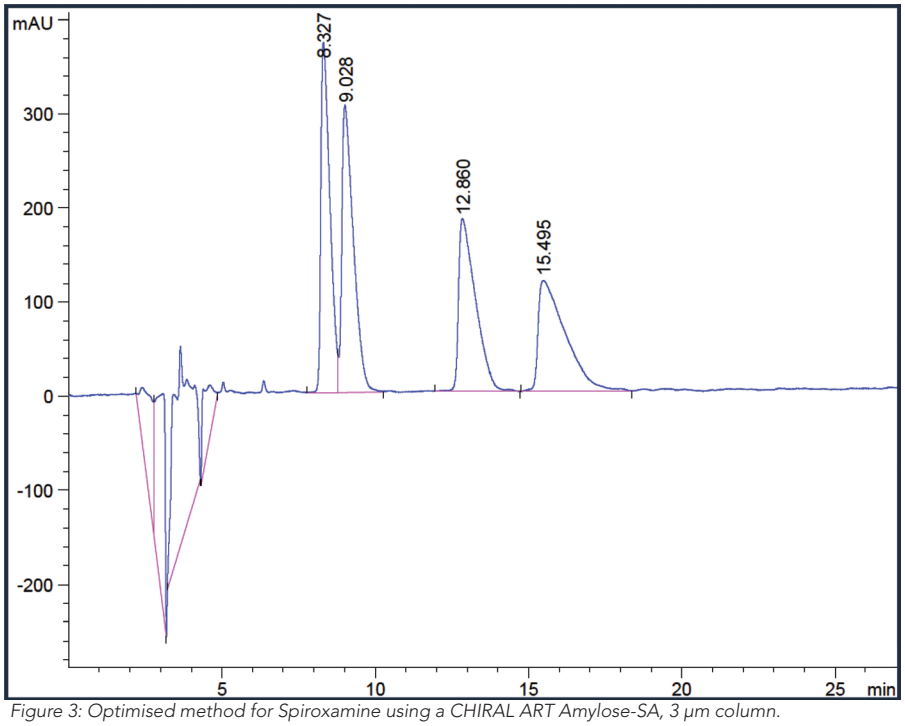

Figure 3: Optimised method for Spiroxamine using a CHIRAL ART Amylose-SA, 3 µm column.

Experimental

For the UV method development, a CHIRAL ART Amylose-SA column (3 µm, 150 x 3 mm ID, YMC Europe, Dinslaken, Germany) was used in an Agilent 1260 HPLC system with UV detector (Agilent, Waldbronn, Germany). The final eluent was water/ethanol/diethylamine (27.5/72.5/0.1), applied at a flow rate of 0.25 mL/min. The detection was performed at a wavelength of 210 nm and at a column temperature of 30°C. The amount injected was 10 µL (10 mg/mL).

The LC-MS/MS instrument consisted of an Agilent 1290 UPLC system (binary and isocratic pump, Agilent, Waldbronn, Germany), a CTC autoinjector (Axel Semrau, Sprockhövel, Germany) and a Sciex API6500 high-end triple-quadrupole mass spectrometer (AB Sciex Germany, Darmstadt, Germany). The eluents used in the LC-MS method were A: water/ethanol 9/1 + 10 mM ammonium carbonate (pH ~9.5) and B: water/ethanol 1/9 + 10 mM ammonium carbonate, which were applied in a ratio of 25/75 (A/B) at a flow rate of 0.3 mL/min. The injection volume was reduced to 1 µL, while the temperature was kept constant at 30°C. A post-column make-up via a T-piece with 0.3 mL/min 1% formic acid in methanol/ water 50/50 was used. Multiple-reaction-monitoring (MRM) mode in ESI positive, MRM 298-144 for quantitation and MRM 298-100 for confirmation was used.

Results and Discussion

Chiral RP Screening

To overcome the requirements of RP conditions, immobilised YMC chiral phases, namely CHIRAL ART Amylose-SA, Cellulose-SB and Cellulose-SC (Table 1), were tested with different RP eluents as described in Table 2. A particle size of 5 µm and a dimension of 250 x 4.6 mm ID were used for each column. Sufficient separation could only be obtained using the CHIRAL ART Amylose-SA column (Figure 2).

Separation in less than 20 minutes

In order to achieve the aim of a separation in less than 30 minutes, the column length and ID were reduced. In addition, 3 µm particles were used instead of 5 µm to increase the

Table 3: Final method parameters

Column	CHIRAL ART Amylose-SA, 3 µm (150 x 3 mm ID)
Eluent	A/B (25/75) A: water/ethanol 9/1 + 10 mM ammonium carbonate (pH ~9.5) B: water/ethanol 1/9 + 10 mM ammonium carbonate
Flow rate	0.3 mL/min
Temperature	30°C
Injection volume	1 µL
Iso pump	post-column make-up via T-piece with 0.3 mL/min 1% formic acid in methanol/ water 50/50
MS/MS conditions	Multiple-reaction-monitoring (MRM) mode in ESI positive, MRM 298-144 for quantitation and MRM 298-100 for confirmation

Example Chromatogram with Enantiomer A1 at LOQ in Grape Matrix Extract

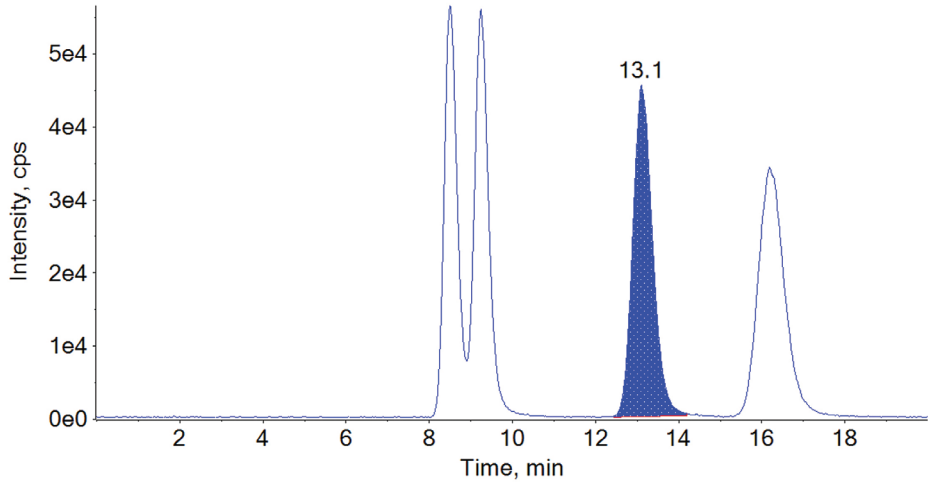

Figure 4: Determination of enantiomer A1 in grape matrix extract; Spiroxamine 10 µg/L (A1, A2, B1, B2 each 2.5 µg/L) [2].

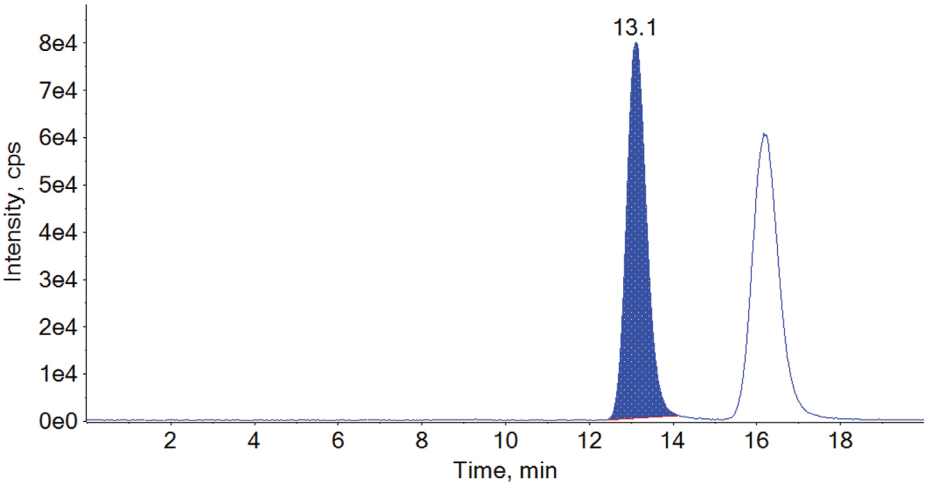

Figure 5: Determination of enantiomer A1 in grape matrix extract; Spiroxamine-A 10 µg/L (A1, A2 each 5 µg/L) [2].

resolution. The ionisation in MS detection can be improved by using an additive, but the addition of acetic or formic acid was found not useful as no retention could be observed.

However, the addition of diethylamine resulted in an even better peak shape. It was not only possible to separate all 4 of the isomers from each other, but also the separation time could be reduced to just 20 minutes. Therefore, all requirements could be fulfilled (Figure 3).

Transfer from LC-UV to LC-MS/MS

It is difficult to compare the UV-method and the final LC-MS method 1:1 as different systems were used for the analysis. The diethylamine used in the UV application was substituted for a 10 mM ammonium carbonate solution (pH 9.5) because the diethylamine strongly influenced the ionisation process and 'quenched' the MS-signal by about 99%. To improve the ionisation 1% formic acid in methanol/ water 50/50 was introduced into the eluent flow post-column changing the pH value from mildly alkaline to mildly acidic (pK 6.9) resulting in the protonation of the Spiroxamine. The resulting, final method parameters are described in Table 3.

Final Set-up

Final conc. at LOQ: 1 µg/L spiroxamine = 0.54 µg/L A isomer; 0.46 µg/L B isomer

= 0.27 µg/L A1 enantiomer

= 0.27 µg/L A2 enantiomer

= 0.23 µg/L B1 enantiomer

= 0.23 µg/L B2 enantiomer

Conclusion

A chiral phase screening using 3 different immobilised columns with different RP conditions was performed to identify the most suitable column to separate the enantiomers of spiroxamine. The selected column, the immobilised CHIRAL ART Amylose-SA, is a silica-based stationary phase modified with the chiral selector amylose tris (3,5-dimethylphenyl-carbamate). As a result of following the method development process all 4 enantiomers could be separated from each other as required. The desired maximum run time of 30 min could even be improved by 10 min, achieving a run time of 20 min.

The eluent used for UV detection, water/ethanol/diethylamine (27.5/72.5/0.1), had to be adjusted for MS detection as the dimethylamine was not suitable. The final eluent system consisted of A: water/ethanol 9/1 + 10 mM ammonium carbonate (pH ~9.5) and B: water/ethanol 1/9 + 10 mM ammonium carbonate at a ratio of 25/75. In addition, formic acid was injected post

Table 4: Chromatographic Performance Parameters

Spiroxamine enantiomer	tR [min]	k'-Value	WH [min]	WH [s]	St.Dev.o [s]	Theor. Plates N	Plate Hight H [µm]	plates/m N/m	Separation Factor an.n+1	Peak Resolution RSn.n+1	Peak capacity n
Enantiomer B1	8.50	2.41	0.3385	20.31	8.628	3,494	42.9297	23,294			29
Enantiomer B2	9.24	2.70	0.3570	21.42	9.099	3,712	40.4083	24,747	1.12	1.22	
Enantiomer A1	13.10	4.25	0.5080	30.48	12.948	3,685	40.7064	24,566	1.57	4.47	
Enantiomer A2	16.19	5.49	0.6708	40.25	17.098	3,228	46.4698	21,519	1.29	2.71	

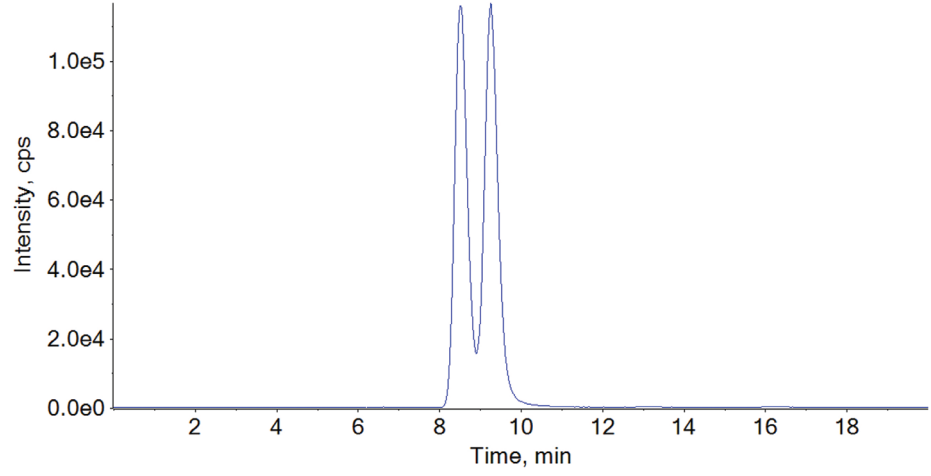

Figure 6: Determination of enantiomer A1 in grape matrix extract; Spiroxamine-B 10 µg/L (B1, B2 each 5 µg/L) [2].

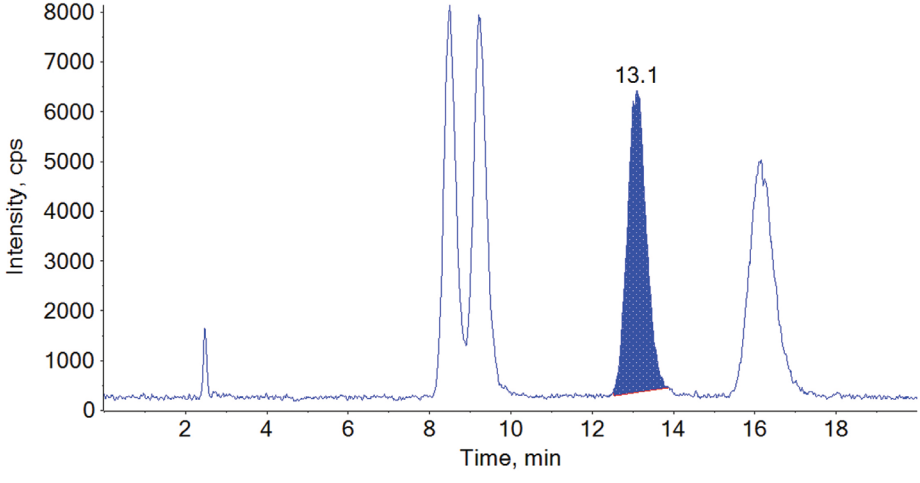

Figure 7: Determination of enantiomer A1 in grape matrix extract; Spiroxamine 1 µg/L in WTFR [2].

column to change the pH to an acidic value.

The resulting, final limits of quantitation were between 0.23 µg/L for the B1/B2 enantiomer and 0.27 µg/L for the A1/A1 enantiomer making the method applicable for detection of trace concentrations. The usability of the method was confirmed by analysing the spiroxamine enantiomers in trace concentrations in grape matrix.

Acknowledgments

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1. Etzel WA, Gau W, Krämer W, Stelzer U, Weissmüller J, Magnetic Resonance in Chemistry, 36 (1998), 64-68.

2. All chromatograms kindly provided by S. Stuke, Bayer AG.